Clonality of smooth muscle and fibroblast cell populations isolated from human fibroid and myometrial tissues

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ABSTRACT: Uterine fibroids are conventionally defined as clonally derived benign tumours from the proliferation of a single smooth muscle cell (SMC). We have previously identified fibroblast-like cells in fibroids, the presence of which raises the question as to whether all cells within the fibroid have the same clonal origin. The first aim of this study was to develop a fluorescence-activated cell sorting (FACS)-based method to isolate different cell types from human myometrium and fibroid tissues. Secondly, we aimed to use X chromosome inactivation analysis to determine the clonality of cell subpopulations isolated from myometrial and fibroid tissues. Human myometrium and fibroid tissues were collected from women undergoing hysterectomy. Immunohistochemistry (IHC) and flow cytometry confirmed that in addition to SMCs, fibroblasts constitute a significant proportion of cells in human myometrium and fibroid tissues. FACS based on CD90 and ALDH1 reliably separated cells into three myometrial and four fibroid subpopulations: SMCs, vascular smooth muscle cells and two fibroblast subsets. Clonality was first determined by X chromosome inactivation using the classic DNA methylation-sensitive HUMARA assay. Data from this assay were highly variable, with only a quarter of samples meeting the definition of clonal fibroid and non-clonal myometrium. However, using an RNA-based X chromosome inactivation HUMARA assay, we were able to demonstrate clonality of all cellular constituents of most fibroids. Our results confirm that most fibroids are derived from a single cell, and for the first time demonstrates that these clonal cells differentiate into fibroblast and SMC subpopulation as the fibroid grows.

Key words: uterine fibroids / clonal

Introduction

Leiomyoma, or fibroids, are benign tumours of the myometrium, and one of the commonest tumours in reproductive aged women (Stewart, 2001; Baird et al., 2003). Despite the burden of uterine fibroids on women and society, the aetiology of these tumours is largely not understood. By conventional pathology, fibroids are typically described as clonally derived from the proliferation of a single clone of smooth muscle cells (SMC) (Kurman, 2002). This definition is based on routine histological observation, as well as evidence for non-random inactivation of the X chromosome as demonstrated by human androgen receptor (HUMARA) or glucose-6-phosphate dehydrogenase (G6PD) isofrom expression (Linder and Gartler, 1965; Mashal et al., 1994; Zhang et al., 2006; Cai et al., 2007). However, the paradigm that fibroids comprise a clonal expansion of SMC ignores the cellular heterogeneity of this tumour (Zhao and Rogers, 2013), as well as the fact that fibroblasts constitute a major cellular component of fibroids that can be recognized by routine IHC and cell sorting (Zaitseva et al., 2007; Moore et al., 2010).

Despite fundamental structural and functional differences between fibroblasts and SMC, virtually all in vitro and gene profiling studies to date have treated fibroid tumours as a homogeneous population consisting of an SMC phenotype. Tumour growth is determined in part by the tumour stroma, which is composed of extracellular matrix (ECM), mesenchymal cells, vessels, nerves and inflammatory cells (Kalluri and Zeisberg, 2006). Fibroblasts produce many of the components of the ECM and contribute to ECM homeostasis and turnover. Therefore, the failure to recognize fibroblasts as distinct functional cell types when investigating the cell and molecular pathology of uterine fibroids is increasingly difficult to reconcile with what is now known about the function, heterogeneity, origin and regulation of fibroblasts (Zhao and Rogers, 2013). In support, functional subsets of fibroblasts can be localized to human myometrium and endometrium, where they are thought to differentially
regulate processes such as inflammation and wound healing in the female reproductive tract (Koumas et al., 2001).

Another tumour initiating cell phenotype recently identified in human uterine fibroids includes somatic stem cell side populations, which possess hallmarks of immature and undifferentiated cells and mesenchymal stem cells (Ono et al., 2007, 2012). These fibroid-derived side population cells, when grown in co-culture with myometrial SMC, are capable of cell differentiation, proliferation in vitro and tumour formation in vivo (Ono et al., 2012). Therefore, consideration of side-population somatic stem cells (SSCs) is also necessary when ascertaining the factors responsible for uterine fibroid growth and development.

The presence of non-SMC in fibroids also raises the important question as to whether all cells within the fibroid have the same clonal origin as first reported in 1965 (Linder and Gartler, 1965). Clonality is routinely examined using the HUMARA assay, which is based on the Lyon hypothesis (Lyon, 1961), where dosage of X chromosome genes are balanced between male and females by inactivation of one X chromosome by hypermethylation during early development in females (reviewed by Escamilla-Del-Arenal et al., 2011). Hence, the aims of this study were as follows: (i) to develop a fluorescence-activated cell sorting (FACS)-based protocol for isolation of different cell populations from human myometrium and fibroid tissues and (ii) to use X chromosome inactivation to determine the clonality of cell subpopulations isolated from human myometrium and fibroid tissues. Our working hypothesis was that one or more fibroblast subpopulations isolated from human fibroid tissues would not be clonally derived.

Materials and Methods

Tissue collection

Human myometrial and fibroid tissues were obtained from women undergoing hysterectomy for fibroids (n = 57, mean age 47.18 ± 0.75 years). Informed consent was obtained from each patient with approval from the Royal Women's Hospital Human Research Ethics Committee or Monash Medical Centre Human Research Ethics Committee. Normal myometrium was taken at least 2 cm from adjacent fibroid tissue. When multiple fibroids were identified, a single sample was taken from the body of the largest fibroid. Patients were not stratified according to fibroid location (subserosal, submucosal or intramural); however, degenerative type fibroids (hyaline, calcification, cystic or red (hemorrhagic)) were excluded. Tissues were either immediately snap frozen and stored at −80°C for nucleic acid extraction, frozen in embedding compound or fixed in formalin for IHC or collected in modified eagle medium (DMEM) culture medium with 10% (v/v) fetal bovine serum (FBS) and antibiotic–antimycotic liquid (Life Technologies, CA, USA) for FACs.

Immunohistochemistry

Paraffin sections (3 μm) were dewaxed and dehydrated. Sections underwent antigen retrieval (boiling citrate buffer pH 6.0, 15 min) (expect for ALDH1 (aldehyde dehydrogenase 1). CD90 IHC was performed on frozen sections (5 μm) and fixed in acetone (5 min). Endogenous peroxidases were blocked with hydrogen peroxide (H2O2) (10 min, room temperature (RT)) (3% (v/v) for paraffin and 0.3% (v/v) for frozen sections). Sections were blocked (10 min, RT) with serum-free protein block (Dako, Glostrup, Denmark). Incubation with primary antibodies occurred overnight at 4°C. For double-IHC, vimentin/caldesmon and vimentin/alpha smooth muscle actin (αSMA), vimentin was visualized with LSAB+ alkaline phosphatase kit (Dako) and vector blue (Vector Laboratories, Burlingame, CA, USA) before application of the next primary antibody (caldesmon or αSMA). Sections were incubated with anti-mouse EnVision+ System-Horse Radish Peroxidase (HRP) (Dako) (30 min, RT), followed by 3,3′-diaminobenzidine (DAB) (Sigma-Aldrich, MO, USA) or 3-amin-9-ethylcarbazole (Life Technologies) (for αSMA only) for 5 min. RT. Single ALDH1 sections were incubated with HRP-anti-goat secondary antibody 1:200 (Life Technologies) (30 min, RT). ALDH1 was visualized with ABC Vectastain kit (Vector Laboratories) (5 min, RT) followed by DAB (5 min, RT). Single-IHC sections (CD90 and ALDH1) were also counterstained with haematoxylin.

Staining protocols for ALDH1/vimentin and αSMA/ALDH1 have been published previously (Zaitseva et al., 2007). Briefly, αSMA/ALDH1 slides were incubated with 3% H2O2 followed by protein block (Dako), and then incubated with αSMA antibody (Dako) (1 h, RT). Secondary antibody incubation followed using the LSAB+ AP kit (Dako) and Vector blue (Vector Laboratories). Slides were then stained for ALDH1 as described above. For ALDH1/vimentin double IHC, slides were stained for ALDH1 as described above, followed by antigen retrieval (boiling citrate buffer). Vimentin staining was performed using the EnVision G2 double stain system (steps 5–8) (Dako) and anti-vimentin antibody (Life Technologies) (1 h, RT). Vector blue was used as a chromagen.

The following primary antibodies were employed; mouse monoclonal vimentin (V9) 1:50 (Life Technologies), mouse monoclonal caldesmon (a-SMA) 1:100 (Dako), mouse monoclonal αSMA 1:400 (Dako), mouse monoclonal CD90 1:1000 (BD Biosciences, NJ, USA) and goat polyclonal ALDH1 1:200 (Santa Cruz Biotecntology, Inc., CA, USA). Isotype controls matched for antibody concentration and species were also conducted. Images were acquired on a Zeiss Axioskop light microscope, AxioCam Icc 3 Zeiss camera and AxioVision System software (Release 4.6) (Carl Zeiss Imaging Solutions, Munich, Germany).

FACS and flow cytometry

Single-cell suspensions of myometrium and fibroid cells were established as described previously (Gargett et al., 2002; Zaitseva et al., 2006). Myometrial and fibroid fibroblasts and SMC populations were separated from endothelial cells, leukocytes and dead cells using five colour FACs. We used CD31 to identify endothelial cells, CD45 to identify leukocytes, CD90 and ALDH for fibroblasts and 4,6-diamidino-2-phenylindole (DAPI) for assessment of cell viability. To detect ALDH1low expressing cells, the ALDEFLUOR™ assay kit was used (StemCells Technologies, VIC, Australia). Cells were re-suspended in PBS/5% FBS (w/v) and incubated with allopurinocyanin (APC)-conjugated CD90 (1:100 or 2 μg/ml, BD Biosciences), phycoerythrin cyanine 7 (PeCy7)-conjugated CD31 (1:100 or 2 μg/ml, BioLegend, CA, USA) and PeCy5.5-conjugated CD45 (1:100, Life Technologies) for 45 min, 4°C. Negative controls were stained with directly conjugated mouse IgG antibodies at equivalent concentrations. Cells were washed, re-suspended in ALDEFLUOR assay buffer and incubated with ALDH substrate BODIPY-aminoacetadaledehyde for 45 min, 37°C. Negative controls were pre-treated with diethylaminobenzaldehyde, a specific inhibitor of ALDH. Cells were pelleted and re-suspended in ALDEFLUOR assay buffer containing DAPI (1 μg/ml, Sigma-Aldrich) for FACs analysis. Cells were sorted on a MoFlo x-cy counter using Cytomation SUMMIT software (Version 4.1; Cytomation, Inc., CO, USA). Cells were selected for analysis based on forward versus side scatter profile; dead cells, leukocytes and endothelial cells were excluded from further analysis based on electronic gating and remaining cells were analysed for ALDH (green fluorescence) and CD90-APC.

For vimentin staining, cells (100,000 cells/tube) were washed in PBS/5% FBS (w/v) and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences). Briefly, cells were suspended in 250 μl of fixation/permeabilization solution, incubated for 20 min at 4°C, washed and re-suspended in 100 μl
permeabilization/wash buffer with 10% (v/v) mouse serum. Cells were incubated with PE-conjugated anti-vimentin antibody (1:10 or 10 μg/ml) for 30 min at 4°C. Cells were washed twice in permeabilization/wash buffer and re-suspended in the same buffer for flow cytometry.

Genomic DNA and RNA extractions
Genomic DNA (gDNA) was extracted from paired myometrium and fibroid tissue using the PureLink Genomic DNA Kit (Life Technologies). For total RNA extractions, paired myometrium and fibroid tissue were homogenized in 1 ml Trizol reagent (Life Technologies) using a PowerLysis 24 bench top bead-beated homogenizer (MO BIO Laboratories, Inc., CA, USA). RNA was further purified using PureLink RNA Mini kit with on-column PureLink DNase treatment (both from Life Technologies). For sorted cells, gDNA and RNA were extracted simultaneously using AllPrep DNA/RNA Micro kit (with on-column DNase treatment for the RNA fraction) (Qiagen, VIC, Australia). RNA/DNA quality and concentration were assessed using a Nanodrop UV spectrophotometer (Thermo Fisher Scientific, VIC, Australia).

DNA-HUMARA
gDNA was digested with HhaI methyltransferase (New England BioLabs, Inc., MA, USA) as described previously (Allen et al., 1992). Briefly, 20 μl reactions containing 200 ng of gDNA for tissue samples or 100 ng of gDNA for sorted cell samples were incubated with 20 U of HhaI for 2 h at 37°C followed by 20 min deactivation at 65°C. Mock digests, without HhaI enzyme were performed simultaneously. Positive control digests using clonally derived placental cells of female origin (a kind gift from Dr Bill Kallionis) and negative control digests, immortalized male fetal foreskin cells (a kind gift from Dr Amy Chui), were included. HhaI digested or mock-digested gDNA (2 μl) was added to 25 μl PCRs reactions with 500 nM human androgen receptor gene (HUMARA) primers (Allen et al., 1992) using GoTaq Green Master Mix (Promega, WI, USA). The forward HUMARA primer was FAM labelled to allow for fragment analysis. Samples were amplified for 30 cycles (tissue) or 35 cycles (cells), each comprising 30 s at 95°C, 60 s at 64°C and 30 s at 72°C, with an initial denaturation at 95°C for 3 min. A second reaction using a different set of HUMARA primers (Mutter and Boynton, 1995) was performed for each sample using the same method (except for annealing 60°C temperature). HUMARA-PCR products underwent capillary electrophoresis separation and fragment analysis (ABI3730 DNA Analyser platform) at the Australian Genome Research Facility (AGR, Melbourne, Australia). X chromosome inactivation [presented as percentage (%) of Allele 1] was calculated from the area under the peak as previously described (Monteiro et al., 1998). A sample was deemed non-clonal if the percentage inactivation fell between ±10 and ±25%, while clonal samples were calculated to have X chromosome inactivation percentages of ±25–50%.

cDNA synthesis and RNA-HUMARA
cDNA from tissues was synthesized from 1 μg of total RNA using High Capacity cDNA Reverse Transcription kit (Life Technologies). cDNA from sorted cells was synthesized from 50 ng of total RNA using VILO cDNA synthesis kit (Life Technologies). HUMARA PCR and peak analysis were performed as described above however, 2 μl of cDNA replaced gDNA in the PCR reaction (Busque et al., 1994; Swierczek et al., 2012).

Statistical analysis
Data were analysed using Graph Pad Prism (version 5, Graph Pad software, CA, USA). Flow cytometry data were analysed by paired t-test, P < 0.05. FACS data were analysed by parametric repeated-measures ANOVA, followed by Tukey’s post hoc test, P < 0.05.

Results

In situ characterization of fibroblast markers
Double immunostaining of SMC markers caldesmon or αSMA, with fibroblast marker vimentin, confirmed a clear distinction in both myometrium and fibroids between SMC (caldesmon and αSMA positive), compared with fibroblasts (vimentin positive) (Fig. 1a-d). In previous studies, CD90 (or Thy1) has been used as a marker for a subset of myometrial fibroblasts (Koumas et al., 2001). In our hands, CD90 appeared to stain fibroblasts more strongly than SMC, while vascular SMCs (VSMC) were negative for CD90 (Fig. 1e–f). Strong fibroblast-like ALDH immunostaining is shown in Fig. 1g–h. ALDH double immunostaining with vimentin confirms this fibroblast-associated phenotype, with populations of ALDH-positive cells also staining positive for vimentin (Fig. 1i–j). However, not all vimentin-positive cells were ALDH positive (Fig. 1l–j). In addition, ALDH-positive cells were negative or very weak for αSMA (Fig. 1k–l).

The proportion of ALDH fibroblasts is lower in fibroids compared with myometrium
To further characterize the fibroblast populations of myometrium and fibroids, we performed flow cytometry for vimentin, CD90 and ALDH on freshly isolated myometrial and fibroid cells. Overall, a greater percentage of cells were positive for CD90 (Fig. 2b), than vimentin (Fig. 2a) or ALDH (Fig. 2c). The percentage of vimentin- and CD90-positive cells were highly variable between samples with no significant difference between myometrium and fibroid tissues for the size of the vimentin and CD90 populations (Fig. 2a and b). In the myometrium, ALDH-positive cells ranged from 14.0 to 60.4% (mean 34.1 ± 6.2%), while in the fibroid ALDH populations ranged from 2.8 to 16.1% (mean 9.8 ± 2.0%) (Fig. 2c). There was a significantly higher percentage of ALDH-positive cells in the myometrium compared with matched fibroid (P = 0.0039) (Fig. 2c). These flow cytometry results are in agreement with vimentin, CD90 and ALDH positivity observed in our IHC (Fig. 1).

Four subpopulations of cells exist in fibroid tissue
Based on our IHC (Fig. 1) and flow cytometry (Fig. 2) results, ALDH and CD90 were selected for double sorting of myometrial and fibroid cells by FACS. Cells were reliably separated into three myometrium and four fibroid subpopulations based on their CD90 and ALDH positivity: ALDH+/CD90- , ALDH+/CD90+, ALDH-/CD90+ and ALDH-/CD90+ (bright) (Fig. 3a-b). The ALDH+/CD90- population was significantly increased in fibroid tissue compared with myometrium (38.0 ± 6.3% fibroid versus 17.9 ± 3.0% myometrium, P < 0.01) (Fig. 3c). In contrast, in fibroid tissue the ALDH+/CD90+ subpopulation was significantly reduced compared with myometrium (10.4 ± 2.2% fibroid versus 35.3 ± 5.9% myometrium, P < 0.001). There was no significant change in the percentage of positive ALDH+/CD90- cells between fibroid and myometrial tissues (15.9 ± 4.8% fibroid versus 17.3 ± 1.2% myometrium) (Fig. 3c). The fourth ALDH+/CD90+ (bright) population was significantly elevated in fibroid tissues, but was absent or substantially reduced in myometrial samples (5.4 ± 1.5% fibroid versus 0.9 ± 0.8% myometrium, P < 0.01) (Fig. 3a and c).
**Fibroid tissue clonality based on the classic gDNA HUMARA assay is highly variable**

The conclusion that uterine fibroids are clonal, and are derived from a single SMC, is based on results examining the non-random inactivation of the X chromosome (Supplementary data, Table S1). Our IHC and flow cytometry data demonstrate that uterine fibroids are composed of cell populations with significantly different phenotypes (Figs 1–3). We therefore decided to investigate the clonality of each cell population. We first examined gDNA from myometrial and fibroid tissues (n = 53 informative patients), using the classic HUMARA assay (Allen et al., 1992). Using the HUMARA assay, clonally derived tissues demonstrate identical patterns of X chromosome inactivation following digestion with Hha1 methyltransferase, interpreted by the loss of one HUMARA allele (Supplementary data, Fig. S2). Surprisingly, our data were highly variable, with only a quarter of samples (24.5% or 13/53) meeting the definition of clonal fibroid and non-clonal myometrium (Supplementary data, Figs S1a and S2a). Almost one-fifth of samples gave precisely opposite than expected results: non-clonal fibroid and clonal myometrium (18.9% or 10/53) (Supplementary data, Fig. S1b and S2b). The largest sub-set of samples (49.1% or 26/53) was classified as non-clonal fibroid and non-clonal fibroid (Supplementary data, Fig. S1d). A small group of four patients (7.5%) were found to have both clonal myometrium and fibroid tissues (Supplementary data, Fig. S1c and S2c).

We validated the assay using a different HUMARA primer sequence (HUMARA2) (Mutter and Boynton, 1995), which significantly correlated with HUMARA1 data in both myometrial tissues ($R^2 = 0.95, P < 0.0001$) and fibroid tissues ($R^2 = 0.96, P < 0.0001$) (data not shown). We also tested the assay by inclusion of control gDNA from clonally derived female and male cells. As expected, following Hha1 digestion, clonal female cells lose a HUMARA allele (Supplementary data, Fig. S2d), while in male cells, only one HUMARA allele is present (Supplementary data, Fig. S2e).

The majority of fibroids are clonal based on expression-associated RNA-HUMARA

An alternative HUMARA method, which allows for clonality determination independent of differential methylation of the X chromosome, was subsequently employed that utilizes RNA instead of DNA (Busque et al., 1994; Swierczek et al., 2012). The RNA-HUMARA assay was used to study 25 paired myometrial/fibroid tissues (also previously analysed by gDNA-HUMARA). The vast majority of fibroid tissues samples (23/25 or 92%) had a severely skewed pattern of X chromosome inactivation, confirming clonal origin of these tumours (Fig. 4). Four women demonstrated both clonal fibroid and myometrium tissue (*), one woman had both non-clonal myometrium and fibroid (†)
Figure 2 Percentage (%) of positive (a) vimentin, (b) CD90 and (c) ALDH cells from single cell suspensions of paired myometrium and fibroid tissues. Flow cytometry was performed on $n = 8–9$ pairs of myometrium and fibroid tissue. Data were analysed by paired t-test, with significant difference denoted by **($P < 0.01$).

Figure 3 Representative FACS plot from (a) myometrium and (b) fibroid, demonstrating gating of four subpopulations: ALDH$^-$/CD90$^-$ (VSMC), ALDH$^-$/CD90$^+$ (SMC), ALDH$^+$/CD90$^{bright}$ (fibroblast) and ALDH$^+$/CD90$^+$ (fibroblast). Subpopulation quantification (c) displayed as percentage (%) of positive cells was determined from $n = 8$ paired myometrial and fibroid FACS runs. Data were analysed by ANOVA (Tukey’s test) and is displayed as mean $\pm$ SEM. Significant differences are denoted by **($P < 0.01$) and ***($P < 0.001$). Proposed cell types of each sub-population are tabled in (d).
and one woman had a non-clonal fibroid and clonal myometrium (‡) (Fig. 4). These data provide a novel means of confirming the clonal origin of uterine fibroids, without the added complication of consideration of DNA methylation heterogeneity.

**Fibroid cellular subpopulations are clonal**

We performed RNA-HUMARA on $n = 8$ sets of ALDH and CD90 FACS sorted cells. Overall, all four fibroid subpopulations exhibited a skewed pattern of $X$ chromosome inactivation and were clonal, except for one population of ALDH$^+$/CD90$^-$ cells in Sample 8 (Fig. 5). The fibroid ALDH$^+$/CD90$^{+\text{bright}}$ population was generally small (refer to Fig. 3c), and we were only able to obtain sufficient RNA from 6 out of 8 patients examined. All of the ALDH$^+$/CD90$^{+\text{bright}}$ fibroid cells were clonal. Both fibroid and myometrial ALDH$^+$//CD90$^{+\text{bright}}$ cell populations were obtained from Sample 4, but only fibroid cells exhibited clonal origin. Approximately half of the myometrial cell populations were also clonal; however, when compared with matched whole tissue, patterns of $X$ chromosome inactivation were consistent between sorted cells and whole tissue. The same was true for fibroid tissues and their constituent sorted cells.

**Discussion**

It is generally accepted that uterine fibroids are benign clonal tumours, derived from the growth of a single uterine SMC (Linder and Gartler, 1965). Our recent studies have questioned this belief (Zaitseva et al., 2007) and in this current study we confirm that uterine fibroids are composed of multiple cell types. FACS sorting myometrium and fibroid for CD90 and ALDH reliably separated tissues into three myometrium and four fibroid subpopulations: ALDH$^+$//CD90$^+$, ALDH$^+$/CD90$^+$, ALDH$^+$/CD90$^+$ and ALDH$^+$//CD90$^{+\text{bright}}$, which based on immunohistochemical cell phenotyping we conclude are enriched for VSMC, SMC, fibroblasts and fibroid-associated fibroblasts, respectively. Although multiple cell phenotypes exist in fibroids, we found that all of these originated from a single clone. The findings of this study extend our current understanding of the basic pathophysiology of uterine fibroids.

Our work supports the conclusion that uterine fibroids are clonal. Since the work of Linder and Gartler (1965) was published, multiple studies have similarly reported that fibroids are clonal (Townsend et al., 1970; Nilbert and Strombeck, 1992; Mashal et al., 1994; Hashimoto et al., 1995; Rogalla et al., 1995; Quade et al., 1997; Baschinsky et al., 2000; Tietze et al., 2000; Canevari et al., 2005; Hui and Fedoriw, 2005; Patton et al., 2006; Zhang et al., 2006; Cai et al., 2007). Most of these studies used methylation-sensitive enzymatic assays which were observational only or analysed semi-quantitatively in conjunction with relatively small sample sizes (10 out of 14 publications report from ≤ 9 patients) (Supplementary data, Table S1). Instances of possible misinterpretation are evident in the literature, for example, failure to acknowledge skewing in normal tissues or the presence of both alleles in tissues deemed to be monoclonal (Mashal et al., 1994; Tietze et al., 2000). Nevertheless, this approach for determining the clonality of fibroid tumours has been universally accepted in the literature; however, in recent years, criticism of $X$ chromosome inactivation clonality assays has grown (Jang and Mao, 2000; Chen and Prchal, 2007; Parsons, 2008; Gomes and Gomez, 2012; Swierczek et al., 2012). Despite the evidence of uterine fibroid cellular heterogeneity and criticism of experimental approach, it is conceivable that the certainty with which the concept of fibroid monoclonality is held reflects the length of time that this theory has gone unchallenged (Parsons, 2008). Of particular concern of late is inaccurate interpretation of the assay and emerging evidence of the complicated and heterogeneous methylation of the HUMARA gene. Methylation is not absolute, with evidence that up to 15% of $X$ chromosome genes escape inactivation and a further 10% are incompletely inactivated (Carrel and Willard, 2005). Extensive variability of HUMARA gene methylation has been demonstrated in clonal single-cell progeny (Swierczek et al., 2012). Abnormal DNA methylation is observed in uterine fibroids and leads to aberrant gene expression, including hypomethylation of HUMARA (Yamagata et al., 2009; Maekawa et al., 2011). Taking into consideration our findings, which does not rely on DNA methylation status, accumulating evidence indicates that...
the traditional gDNA-based HUMARA assay is not well suited for clonality determination in uterine fibroids. The RNA-HUMARA method is an advancement of traditional DNA-HUMARA (Busque et al., 1994; Swierczek et al., 2012). In support, Swierczek et al. (2012) also observed discordance between DNA-HUMARA and RNA-HUMARA, further demonstrating that HUMARA gene methylation does not always correlate with its expression.

This work confirms that fibroids are not composed of a single cell type, and that fibroids are clonal, indicating that the unique clonal subpopulations are differentiating after fibroid establishment. Recent investigations have identified side-population SSCs from uterine fibroids with unique cellular characteristics that distinguish them from differentiated fibroid SMC (Chang et al., 2010; Mas et al., 2012; Ono et al., 2012). Similar to the fibroid-derived fibroblasts we identified in our system, SSCs were CD90-positive (94–99%), indicating that although these cells are of undifferentiated lineage they have a committed mesenchymal phenotype (Mas et al., 2012). We identified that ALDH-associated fibroblasts from uterine fibroids were αSMA negative; fibroid-derived side-population cells also expressed low levels of αSMA (Ono et al., 2012). In vitro, fibroid SSC can differentiate into specific mesenchymal lineages and regenerate into fibroid-like tissue in vivo (Mas et al., 2012; Ono et al., 2012). It has been hypothesized that soluble trophic factors secreted by certain stem cell populations exert potent paracrine actions on other cells and may promote tumour growth (reviewed by Baraniak and McDevitt, 2010). Signals generated from the microenvironment immediately surrounding the clonally derived fibroid cells may also impact on differentiation, where the fibroid subpopulations may be differentiating to mimic the surrounding myometrial niche. Therefore, there is evidence that a population of multipotent SSCs reside in an undifferentiated state in the uterus and have the capacity to differentiate and secrete

**Figure 5** RNA-HUMARA was performed on a set of eight myometrial and fibroid samples following FACS sorting on ALDH and CD90. ALDH+/CD90− VSMC (A−C−), ALDH+/CD90− SMC (A−C−), ALDH+/CD90− fibroblasts (A+C−) and ALDH+/CD90−bright fibroblasts (A+C−bright). Subpopulations were also compared with the percentage X chromosome inactivation from matched whole tissue. X chromosome inactivation (%) between +0 and 25% was deemed non-clonal, while inactivation (%) between ±25 and 50% was classified as clonal.

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The percentage X chromosome inactivation was calculated for each subpopulation, with +0 and 25% being non-clonal and ±25% to 50% being classified as clonal.
paracrine factors that could initiate fibroid tumour growth. We propose that the four subpopulations identified in uterine fibroids are clonally amplified from a multipotent progenitor and that differentiation into SMCs, VSMCs and fibroblast cells occurs after fibroid formation in attempt to mimic the myometrium.

The selective expansion of ALDH−/CD90+ fibroblasts in uterine fibroids may contribute to fibroid growth and development, and furthermore, the different fibroblast-like populations identified in the present study in myometrium and fibroid tissues would likely possess different functions. Individual fibroblast populations have differential functionality with tissue- and response-specific differences being identified. We previously described the presence of fibroblast-like cells in uterine fibroids with a different phenotype from myometrial fibroblasts (Zaitseva et al., 2007). In a separate study using different cell markers, Moore et al. (2010) extracted fibroblast cells from uterine fibroids and also showed that fibroblasts represent a significant cellular component of the tissue. Fibroblast cells are present in human myometrium with confirmation of different fibroblast subpopulations (Thy 1+ and Thy 1−) (Koumas et al., 2001; Chang et al., 2010). In myometrium, Thy 1+ and Thy 1− fibroblasts demonstrate unique phenotypes and respond differently to pro-inflammatory challenge (Koumas et al., 2001). It is believed that only particular fibroblast subsets within a tissue will demonstrate exaggerated proliferative/fibrogenic responses (reviewed by Stenmark et al., 2006), and may be a potential characteristic of the fibroid-specific ALDH−/CD90+ bright cells.

The ALDH−/CD90+ cell population (enriched for SMC) was the largest subset in uterine fibroids, followed by ALDH−/CD90− (enriched for VSMC). We and others have previously used a lack of ALDH to distinguish SMCs (Ginestier et al., 2007; Zaitseva et al., 2007; Penumatsa et al., 2010). However, unlike other groups (Chang et al., 2010), we were able to identify distinct CD90-positive and CD90-negative cells in both myometrium and fibroid tissues with proposed VSMCs distinguished from SMCs based on CD90 negativity. Placental arterial SMCs (Leik et al., 2004) and HUVECs (Kisselbach et al., 2009) are also negative for CD90. ALDH−/CD90− populations were similar in number between myometrium and fibroid tissues. We had expected ALDH−/CD90− VSMC to be non-clonal, since tumour vessels are generally thought to develop from pre-existing vessels in adjoining healthy myometrium. However, in the patients we examined fibroblast-derived ALDH−/CD90− were mostly clonal (7/8 women). Tumour vessels may not always be composed of endothelial cells, and may be surrounded by tumour cells or a mixture of normal and abnormal cells, referred to as ‘tumour cell-generated vascular channels’ (Maniatis et al., 1999; reviewed by Carmeliet and Jain, 2000; Pezzolo et al., 2007). Therefore, it is possible that functional vascular support in tumours can originate from the tumour itself, and we suggest that ALDH−/CD90− cells might arise in this manner.

In conclusion, we confirm that uterine fibroblasts are composed of various cell types: ALDH−/CD90+, ALDH−/CD90−, ALDH+/CD90+ and fibroid-associated ALDH−/CD90+bright, with these populations being enriched for SMCs, VSMCs, fibroblasts and fibroid-associated fibroblasts, respectively. We employed a highly sensitive and quantitative technique to demonstrate that uterine fibroblasts, and their cellular constituents, are clonal in origin. This suggests that the four fibroblast subpopulations may be clonally expanded from a multipotent progenitor cell that undergoes differentiation post-fibroid formation. The identification of distinguished cell populations from uterine fibroids could provide a means of studying fibroid pathophysiology in greater detail.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors’ roles

All authors (S.J.H.-C., M.Z., B.J.V. and P.A.W.R.) contributed to design of the study and S.J.H.-C. and M.Z. were involved in acquisition of data. All authors contributed to analysis and interpretation of data, writing the article and gave their final approval of the article.

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Conflict of interest

None declared.

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